Functionality of a *Bacillus cereus* biological agent in response to physiological variables encountered in *Cyprinus carpio* aquaculture

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Keywords *Bacillus cereus*, pH, salinity, temperature, aquaculture, *Cyprinus carpio*

Abstract Modern aquaculture utilises intensive reticulated systems resulting in waste accumulation and proliferation of disease. Conventional chemical treatments cause resistance in pathogens and negative environmental impact. The potential of a *Bacillus cereus* isolate (NRRL 100132) as a biological agent for aquaculture has been demonstrated in vitro and in vivo. The functionality of this isolate across a range of physiological conditions, including salinity, pH and temperature, based on rearing of high-value ornamental *Cyprinus carpio,* was investigated. Temperature had a significant influence on germination, specific growth rate and increase in cell number of *B. cereus* in shake-flask cultures, while salinity and pH did not have a measurable effect on growth. Controlled studies in bioreactors and modelling of the data to the Arrhenius function indicated the existence of high and low growth temperature domains. The rates of pathogenic *Aeromonas hydrophila* suppression and decrease in waste ion concentrations (ammonium, nitrite, nitrate, phosphate) were translated into a linear predictive indicator of efficacy of the *B. cereus* isolate at different temperatures. The present study confirmed the robustness of the *B. cereus* isolate (NRRL 100132) as a putative biological agent for aquaculture and further demonstrated a novel method for the assessment of in vitro biological efficacy as a function of temperature.

Introduction

Success of modern aquaculture is driven by intensive reticulated culture systems (Liao and Mayo 1974), whereby high growth rate and high stocking density are major requirements. However, this practise results in the onset of fish disease and environmental pollution (Paperna 1991). The rearing of ornamental *Cyprinus carpio* (koi) is a rapidly growing, high value industry and the health and survival of ornamental carp is thus an important requirement for both hobbyists and culturists.

Carp are prone to a wide range of diseases with one of the major causative agents being *Aeromonas hydrophila*, which results in the outbreak of bacterial ulcer diseases by haemorrhagic septicaemia (Austin and Austin 1999; Jeney and Jeney 1995). Since mass mortality can occur if there is a prevalence of infectious agents (Irie et al. 2005), reducing the prevalence of bacteria such as *Aer. hydrophila* in water systems used to rear ornamental carp is required. Fish disease is normally a consequence of the interaction between the host, environmental stress elements and disease causing agents (Austin and Austin 1999; Jeney and Jeney 1995). The deterioration of water quality as a result of metabolic waste accumulation (ammonia, nitrite, nitrate and phosphate) should thus be considered (Jana and Jana 2003). The main source of metabolic waste is through excessive feeding rates (Boyd 1985; Liao and Mayo 1974) and dietary composition (Kim et al. 1998; Shimeno et al. 1997), which is amplified in ornamental carp systems due to the use of high nutrient diets. Addition of fresh water, to decrease concentrations of waste metabolites, causes effluent purges resulting in negative environmental impact. Conventional methods such as disinfectants and chemotherapeutics have been used to treat bacterial disease however these result in development of resistance in pathogenic organisms and chemical residues with

detrimental effects to end users and the environment (Barker 2000; de Kinkelin and Michel 1992; Jana and Jana 2003; Moriarity 1999; Sze 2000; Verschuere et al. 2000).

Bacterial amendments have emerged as an appropriate alternative to the use of antibiotics in aquaculture (Hong et al. 2005; Vanbelle et al. 1990) and have demonstrated the potential to improve water quality, decrease pathogen load and reduce fish mortality (Fast and Menasveta 2000; Jana and Jana 2003; Moriarity 1999; Skjermo and Vadstein 1999). Gram positive *Bacillus* species are an attractive option as a bacterial amendment for aquaculture as these organisms are found naturally in sediments, are ingested by animals and are unlikely to use genes for antibiotic resistance or virulence from Gram negative organisms such as *Aeromonas spp*. (Moriarity 1999). Furthermore, bacterial spores of the genus *Bacillus* have several advantages compared to vegetative cells, such as resistance to toxic compounds, temperature extremes, desiccation and radiation (Wolken et al. 2003), thus allowing for the formulation of stable products (Hong et al. 2005; Ugoji et al. 2006). Several spore forming bacteria such as *B. coagulans, B. subtilis, B. clausii, B. cereus* and *B. toyoi* are already exploited as components of products for human and animal use (Sanders et al. 2003).

A natural isolate of *Bacillus cereus* (NRRL 100132) was shown to inhibit pathogenic *Aer. hydrophila* and to decrease the concentrations of ammonia, nitrite, nitrate and phosphate ions in previous in vitro and in vivo studies (Lalloo et al. 2007). Application of biological agents requires demonstration of the tolerance of the micro-organism to the conditions in which they must perform (Gross et al. 2003; Moriarity 1999), in particular their ability to germinate and grow (Wolken et al. 2003). The functionality of a biological agent is often

affected by physiological conditions such as salinity, pH and temperature, and biological agents must remain functional and effective over a wide range of harsh physiological conditions (Guetsky et al. 2002). The benefits of bacterial additives to water and the tolerance of biological agents to the physiological conditions of the environment in which they are applied have not been sufficiently demonstrated (Fast and Menasveta 2000), due to the lack of in vitro investigation of the interaction between key physiological variables. This is the first reported study of the effects of key physiological variables on the functionality of a putative biological agent targeted for use in the rearing of ornamental *C. carpio.*

Materials and methods

Micro-organisms and inocula

The *B. cereus* (NRRL 100132) isolate (Lalloo et al. 2007) was cultured in medium containing 0.8%m.v^{-1} Yeast Extract, 0.005%m.v^{-1} $MnSO_4$, 0.01%m.v^{-1} $CaCl_2$ and 0.03%m.v^{-1} $MgSO_4.7H_2O$ for 24 hours at 30^{o}C, pH 7.0 and 180 RPM in an orbital shaker (Innova 2300, New Brunswick Scientific, Edison, NJ). *Aer. hydrophila* (ATCC 7966), a fish pathogen, was similarly cultured on selective media (Kielwein et. al. 1969; Kielwein 1971). Both cultures were inoculated from cryo-preserved cell banks prepared according to Meza et al. (2004). Resultant cultures were used as inocula for experiments. All materials used in the present study were obtained from Merck (Darmstadt, Germany), unless otherwise specified.

Cultivation and media

Cultivation of *B. cereus* in shake-flasks over a range of salinity, pH and temperature were performed according to a Box Behnken matrix experimental design, followed by statistical analysis of the data using Design Expert-6 software (Stat-Ease, Inc., Minneapolis, USA). The ranges for the numeric factors conformed to the tolerable ranges for rearing of ornamental *C. carpio* (Lammens 2004), whereby NaCl between 0 to 1% $m.m^{-1}$, pH between 6 to 9 and temperatures between 4 to 30^{o}C were tested. Shake-flask experiments were conducted in activation media (0.075% $m.m^{-1}$ spray dried corn steep liquor and 0.025% $m.m^{-1}$ Dextrose monohydrate) developed previously (data not presented), and supplemented with NaCl according to the experimental design. Spray dried corn steep liquor was obtained from Roquette (Lestrem, France). The pH of the media was adjusted to the desired value using either 20% $m.v^{-1}$ NaOH or 10% $m.v^{-1}$ H_2SO_4. Media was prepared, inoculated and incubated as described previously (Lalloo et al. 2007), at the desired temperatures. Experiments were sampled two hourly. Specific growth rate (μ), germination ratio and increase in viable cell number were the responses measured.

Cultivation of *B. cereus* across a range of temperatures (4, 13, 16, 20, 25 and 30^{o}C) was conducted in Braun Biostat B fermenters (Sartorius BBI Systems, Germany). The activation media was prepared to a volume of 1400 mL in the fermenter and sterilized at 121^{o}C for 30 minutes. The pH of the media was adjusted in situ to 7.5 with 20% $m.v^{-1}$ NaOH, followed by inoculation with *B. cereus* culture to result in an initial concentration of 1 x 10^5 $CFU.mL^{-1}$. The airflow was maintained at 1 $v.v^{-1}.m^{-1}$ and agitation at 300 rpm to ensure oxygen saturation relative to ambient conditions. The reactor was sampled four hourly.

Specific growth rate (μ), germination ratio and increase in viable cell number were measured.

Shake-flask evaluation of efficacy of *B. cereus* across a range of temperatures was also performed in filter sterilized Synthetic Pond Water (0.0085% $m.v^{-1}$ KNO_3; 0.006% $m.v^{-1}$ $NaNO_2$, 0.0093% $m.v^{-1}$ $(NH_4)_2SO_4$, 0.0038% $m.v^{-1}$ H_3PO_4, 0.1% $m.v^{-1}$ Yeast Extract and 0.1% $m.v^{-1}$ Glucose), to mimic conditions in rearing ponds for *C. carpio*. Each shake-flask was co-inoculated with *B. cereus* and *Aer. hydrophila* to an effective final concentration of approximately 1.0×10^5 $CFU.mL^{-1}$ for each micro-organism except for control flasks which only contained *Aer. hydrophila*. Flasks were sampled two hourly and analyzed for viable cell number of *Aer. hydrophila* and *B. cereus* and ammonium, nitrite, nitrate and phosphate concentrations.

Analyses and Calculations

All experiments were conducted in triplicate. The specific growth rate (μ) was determined from OD_{660nm} measurements (Genesys 20, Spectronic, USA) for data points conforming to high linearity ($r^2 > 0.9$) of a plot of ln (OD_{660nm}) against time. Viable cell counts were determined by spreading serially diluted samples of *B. cereus* onto nutrient agar plates and *Aer. hydrophila* onto selective agar plates (Kielwein et al. 1969; Kielwein. 1971) followed by incubation at 30^oC for 24 hours and enumeration of colony forming units. The increase in cell number was quantified by difference in viable cell counts between final and initial samples. Germination ratio, which is an indication of the percentage of vegetative cells within the culture, was calculated as the ratio between the vegetative cell concentration and total cell concentration (Monteiro et al. 2005) as determined by microscopic counting of

cells and spores using a Thoma counting slide (Hawksley and Sons, London, UK). Ammonia was analyzed using a Reflectoquant (Catalogue No. 1.16892.0001, Merck, Darmstadt, Germany), and nitrate, nitrite and phosphate ion concentrations were measured by ion chromatography (Morales et al. 2000) using an Ion Chromatography System (Dionex, Sunnyvale, USA) with an anion pre-column and anion separator column (Dionex AG14 and AS14, Sunnyvale, USA). The rates of decrease in the concentration of ammonium, nitrite, nitrate and phosphate ions were determined by using data points conforming to high linearity ($r^2 > 0.9$) of plots of ion concentration against time. An Arrhenius plot was generated by plotting the ln function of maximum specific growth rate against the reciprocal of the absolute temperature (^oK). The square root of maximum growth rate was plotted against the absolute temperature (^oK) to examine conformance of the data to the Ratkowsky function (Choma et al. 2000; Ratkowsky et al. 1983). The normalised efficiency of the biological agent was calculated by determination of relative percentage values using the linear equations for the actual rates of decrease in concentration of waste ions and *Aer. hydrophila*, with the rate at 25^{o}C (optimum temperature for rearing of ornamental *C. carpio*) being equated to 100% efficiency.

Results

Effect of temperature, salinity and pH on growth of *B. cereus*

The effects of temperature, salinity and pH on spore germination and cell growth of the putative biological agent *B. cereus* during shake-flask cultivation in activation medium are presented in Figure 1. Changes in temperature demonstrated a significant impact on

germination ratio ($p<0.0001$), increase in cell number ($p<0.01$) and specific growth rate ($p<0.001$) of the biological agent. Decreasing the temperature from 30 to 4^{o}C resulted in a drastic decline in germination ratio from 95 to 29%, viable cell number from 4.6 x 10^{7} to 3 x 10^{1} CFU.mL^{-1} and specific growth rate from 0.718 to 0.035h^{-1} respectively. However, there was no significant impact of salinity or pH on the responses measured within the minimum-maximum of the ranges tested ($p>0.1$).

The impact of temperature on *B. cereus* growth was further investigated under well controlled conditions in bioreactors (Figure 2). The germination ratio was lowest at 4^{o}C (28.8%) and increased substantially up to 13^{o}C (83.2%). The further increase in germination ratio at temperatures between 13^{o}C and 30^{o}C was negligible resulting in an average germination ratio of 88.5 ± 4.9 %. A rapid increase in cell number was observed between 4^{o}C to 20^{o}C, while higher cultivation temperature did not significantly increase cell number ($p=0.29$). The correlation between temperature and specific growth rate could be modelled using a linear function ($\mu = 0.027t - 0.1052$; $r^2 = 0.99$), which could be used to estimate the specific growth rate of the biological agent at any given temperature. The growth rate response to temperature was also modelled to the Arrhenius and Ratkowsky functions (Figure 3). The Arrhenius plot resulted in a lower linear regression of the complete data set ($r^2 = 0.92$), while separate linear regressions for each of the low and high temperature data domains were highly significant ($r^2 = 0.99$). The growth rate data also conformed to the Ratkowsky function ($r^2 = 0.99$), indicating that the growth rate response of the biological agent to temperature is similar to that of other micro-organisms.

Effect of temperature on the functionality of *Bacillus cereus* as a biological agent

The impact of temperature on the efficacy of *B. cereus* as a biological agent for aquaculture, measured as the decrease in concentrations of pathogenic *Aer. hydrophila* and ammonium, nitrite, nitrate and phosphate waste ions, was investigated at 13, 20 and 30^{o}C in shake-flasks using Synthetic Pond Water. These conditions mimic the environments in rearing ponds for *C. carpio* and conformed to the low, midpoint and high temperature domains for *B. cereus* growth as determined from the Arrhenius plots. Co-culturing in the presence of pathogenic *Aer. hydrophila* resulted in *B. cereus* growth rates of 0.424, 0.579 and 0.718 h^{-1} at 13, 20 and 30^{o}C, respectively (Figure 4). Although the *Aer. hydrophila* growth rate in control treatments (absence of *B. cereus*) was also reduced at lower temperature, a marked decrease in Aer. *hydrophila* cell number was observed during *B. cereus* co-culture (Figure 5). The *B. cereus* biological agent therefore reduced the *Aer. hydrophila* growth at all the temperature points tested. The difference in viable cell number of *Aer. hydrophila* between control and test treatments at the endpoint of the study was 3.5, 6.9 and 9.7 x 10^5 CFU.mL^{-1} at 13, 20 and 30^{o}C respectively, confirming that the reduction in *Aer. hydrohila* cell number is attributed to the actual presence of *B. cereus*. Significant rates of decrease in the concentrations of ammonium, nitrite, nitrate and phosphate waste ions were observed across the range of temperatures studied, as a result of *B. cereus* co-culture (Fig. 6).

The rate of decrease in concentrations of *Aer. hydrophila* and ammonium, nitrite, nitrate and phosphate ions could be correlated to the cultivation temperature in a linear manner (r^2 > 0.98; Figure 6a), resulting in equations that could predict the efficacy of the biological agent against each of these criteria. A more useful indicator of efficacy of the biological

agent at different temperatures was determined by normalisation of the rates of decrease to a temperature equivalence point (25^{o}C being the optimum temperature for rearing of ornamental *C. carpio*), thus resulting in a single straight line equation (R_{nr} = 6.691*t* – 67.461; r^2 = 0.99) for prediction of the efficacy of the biological agent across different temperatures (Figure 6b).

Discussion

There is sufficient evidence of the benefits associated with the use of spore forming bacteria, such as *Bacillus spp.*, as biological agents for improving water quality and reducing disease in aquaculture (Gomez-Gil et al. 2000; Hong et al. 2005; Irianto and Austin 2002; Lalloo et al. 2007; Rengipat et al. 2000; Sanders et al. 2003; Wolken et al. 2003; Vaseeharan and Ramasamy 2003). *B. cereus* (NRRL 100132) was previously demonstrated as a putative biological agent for potential commercial use in culture of ornamental *C. carpio* (Lalloo et al. 2007). Information on the suitability and robustness of putative biological agents in response to environmental conditions such as salinity, pH and temperature are limited, yet changes in these conditions influence cell growth, survival and functionality of *Bacillus spp*. as biological agents (Budde et al. 2006). The functionality of the *B. cereus* NRRL 100132 isolate across the extreme ranges of culture conditions for *C. carpio* was therefore investigated. Oxygen was not considered as it is generally maintained at ambient saturation conditions in carp rearing systems, while an in vivo study did not indicate any negative impact of the biological agent on oxygen concentration (Lalloo et al. 2007).

The present study has demonstrated that the growth of the *B. cereus* biological agent could be maintained across the range of pH and salinity typically applied to the rearing of *C. carpio*, while changes in temperature had a significant impact on spore germination and vegetative cell growth (Figure 1). The absence of interaction between salinity (NaCl concentration), pH and the growth of a different strain of *B. cereus,* within the ranges tested in the present study, has been reported previously (Chorin et al. 1997; Jobin et al. 2002; Leguerinel et al. 2000).

Spore germination, growth rate and increase in cell number of *B. cereus,* were low at 4^{o}C but increased substantially above 13^{o}C (Figure 2). Germination was however observed at 4^{o}C in contrast to the study by Chorin et al. (1997), where no growth was observable at this temperature using different strains of *B. cereus*. Cell number increased significantly up to 20^{o}C, with limited increase thereafter, indicating a maximum cell yield on the growth media independent of the temperature increase from 20^{o}C to 30^{o}C. *Bacillus subtilis* was also shown to sustain viability below 11ºC (Nicholson et al. 2000) and cold shock responses have been furthermore observed below these temperatures (Budde et al. 2006). The growth rate data was indicative of a thermo-kinetic relationship with increasing temperature (Figure 2c). When modelled to the Arrhenius function, low and high temperature domains were observable (Figure 3a) which conformed to changing metabolic growth rates between the low and high ranges respectively. A similar observation has previously been reported for *B. cereus* TZ415, where the critical switch point was 13^{o}C (Choma et al. 2000). The experimental data showed a linear correlation to the Ratkowsky function (Figure 3b) indicating that the model is useful as a predictive tool of growth rate for *B. cereus* (NRRL 100132) across a temperature range. In contrast to the Arrhenius model however, the temperature domains could not be predicted by the Ratkowsky

function, thus demonstrating the usefulness of the Arrhenius function in predicting the growth of biological agents in vitro.

Attenuation of the growth rate of the *B. cereus* biological agent at lower temperatures did not translate directly into a lack of functionality, since acceptable rates of pathogen suppression and removal of waste metabolites across the range of temperature was observed. Furthermore, the metabolic rate of *C. carpio* is significantly reduced at lower temperatures, which translates to a reduced intake of feed, waste metabolite generation and concomitant decrease in pathogen propensity (Lammens 2004). The present study also demonstrated a reduction in growth rate of pathogenic *Aer. hydrophila* at low temperature. Attenuation of pathogen growth by *B. cereus* increased with increasing temperature (Figure 6) indicating that the functionality of the biological agent was growth associated and could potentially be ascribed to the mechanism of competitive exclusion (Sanders et al. 2003; Hong et al. 2005). The decreases in concentrations of waste ions were also enhanced at higher temperatures. Nitrogen removal is classically ascribed to autotrophic bacteria in natural systems, but there have been several reports suggesting a contribution by heterotrophic bacteria in this regard (Kim et al. 2005; Lin et al. 2006; Martiensen and Schöps 1999; Robertson and Kuenen 1990; Sakai et al. 1996; Sakai et al. 1997; Su et al. 2001).

The present study has demonstrated a novel method for assessment and prediction of the functionality of biological agents in an in vitro system across a temperature range. Results of the in vitro assay of the *B. cereus* isolate for disease control and water quality

enhancement in the rearing of ornamental *C. carpio* were in agreement with an in vivo assessment using ornamental *C. carpio* in a previous study (Lalloo et al. 2007). Other researches have also reported that the addition of beneficial bacteria can enhance the health of animals by effecting a holistic improvement in waste ion removal and pathogen reduction (Boyd and Tucker 1998; Frances et al. 2000; Jeney and Jeney 1995; Larmoyeux and Piper 1973; Liao and Mayo 1974; Shimeno et al. 1997). By normalizing the rates of decrease of pathogenic *Aer. hydrophila*, ammonium, nitrite, nitrate and phosphate relative to the optimum temperature (25^{o}C) for growth of *C. carpio* (Metz et al. 2003), a useful predictive model was generated which expresses the holistic functionality of the biological agent across a temperature range.

Acknowledgements

Nodumo Zulu is acknowledged for technical assistance.

This work was supported by BioPAD Biotechnology Regional Innovation Centre.

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Figure Legends

Figure 1 Growth rate, cell number increase and spore germination in response to [NaCl], pH and temperature in matrix studies during shake-flask cultivation

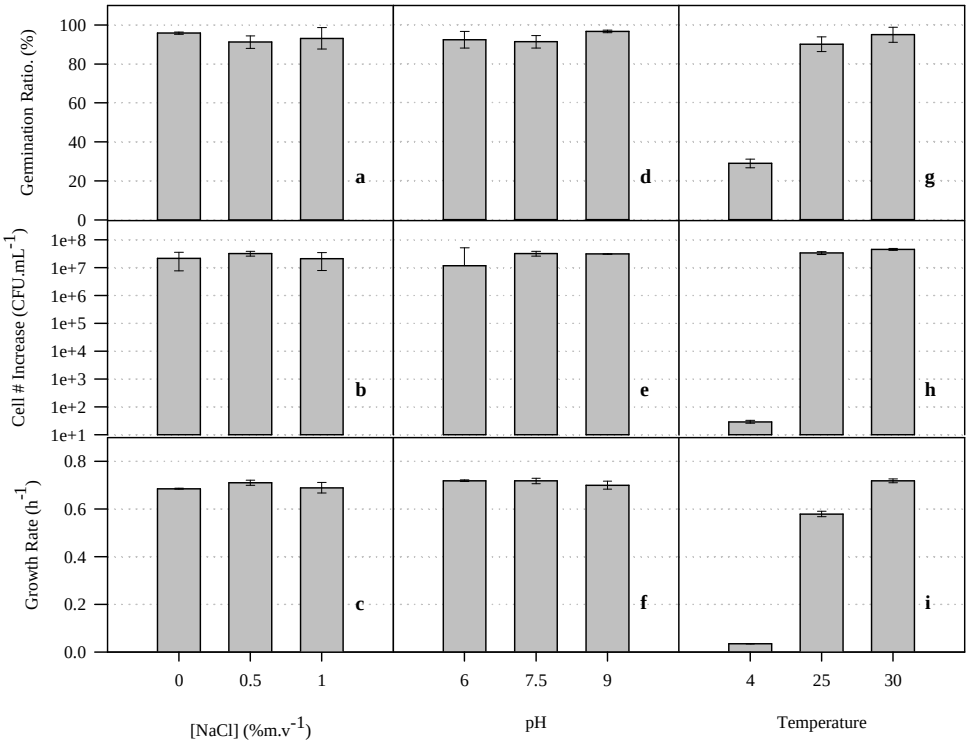
Figure 2 Growth rate, cell number increase and spore germination in response to temperature in bioreactor cultivation

Figure 3 Arrhenius and Ratkowsky plots in response to temperature

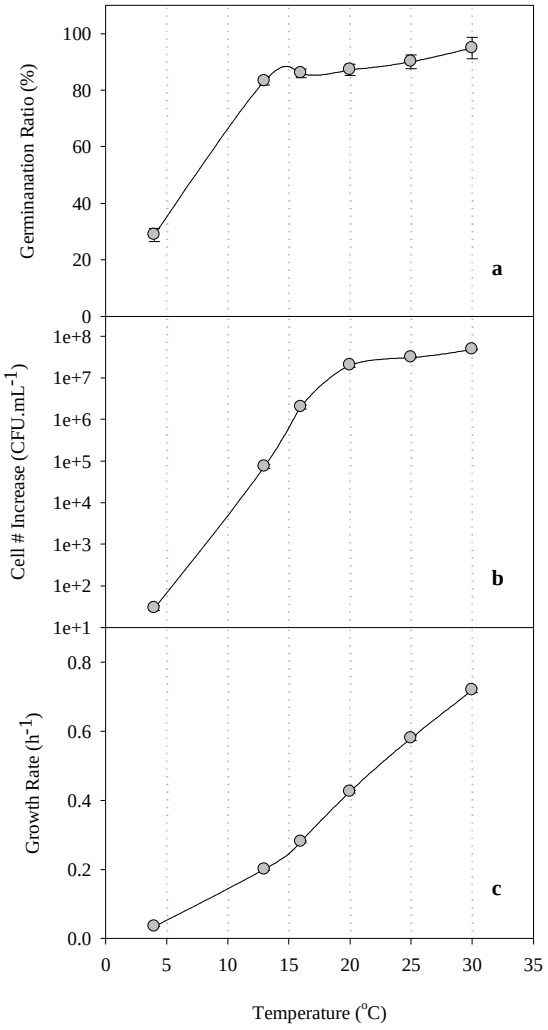
Figure 4 Growth of *B. cereus* in co-culture with *Aer. hydrophila* at low, mid and high temperature domains in shake-flask culture

Figure 5 Growth of *Aer. hydrophila* in co-culture with and without *B. cereus* at low mid and high temperature domains in shake-flask culture

Figure 6 Rate of decrease in pathogen and waste ion concentrations in response to temperature


Germination Ratio. (%)
Cell # Increase (CFU.mL-1)
Growth Rate (h-1)
a
b
c
d
e
f
g
h
i
[NaCl] (%m.v-1)
pH
Temperature

1


Germinanation Ratio (%)
100
80
60
40
20
0
a
Cell # Increase (CFU.mL-1)
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
1e+2
1e+1
b
Growth Rate (h-1)
0.8
0.6
0.4
0.2
0.0
c
0
5
10
15
20
25
30
35
Temperature (°C)

2

3

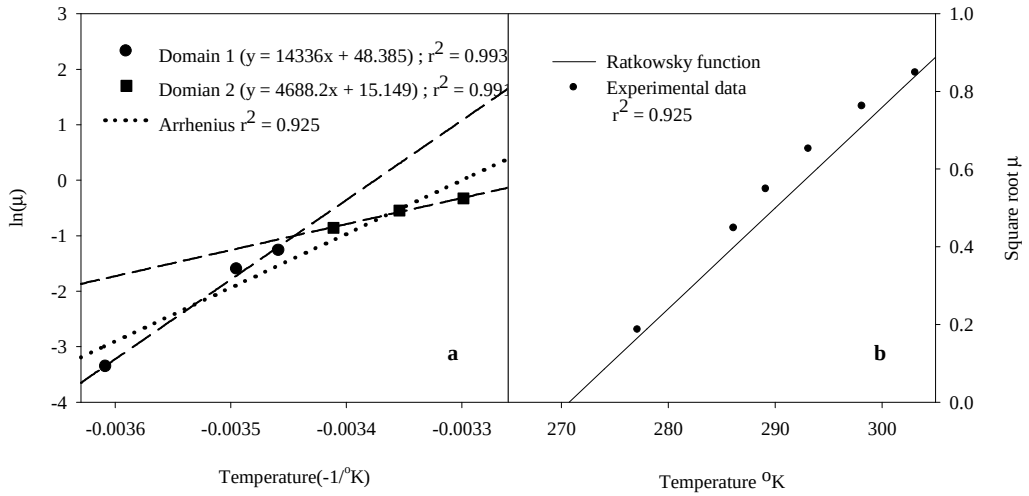
4

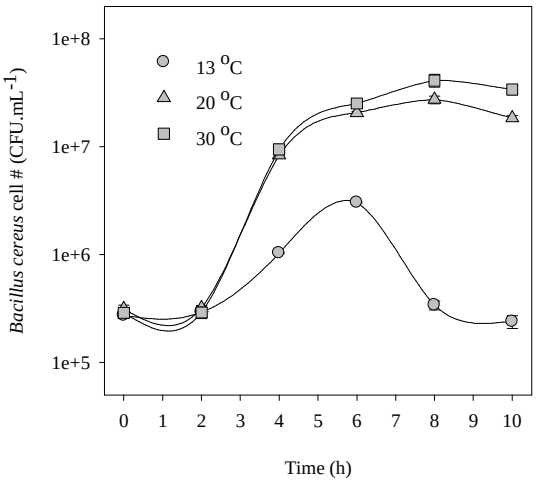
5

6

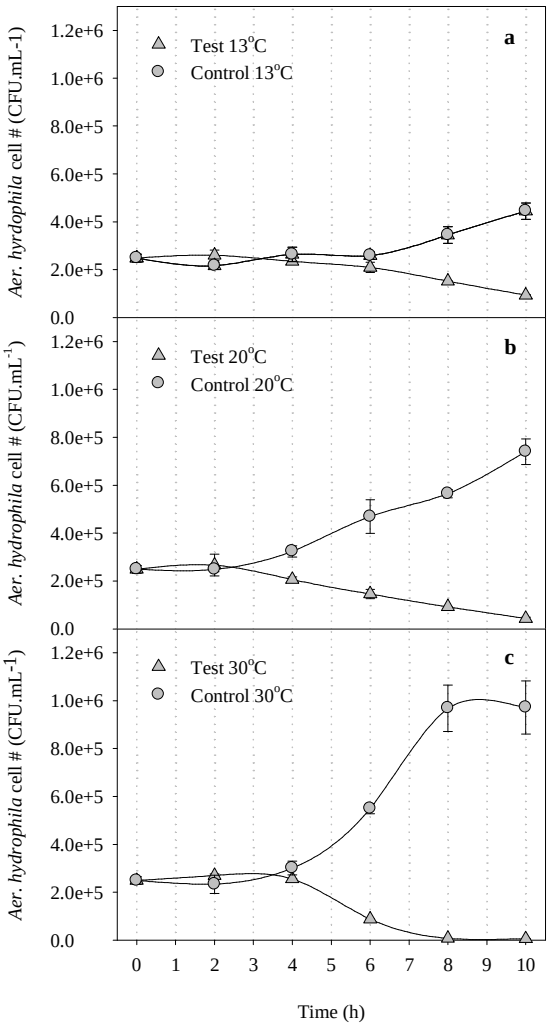
7

8


Domain 1 (y = 14336x + 48.385) ; r2 = 0.993
Domian 2 (y = 4688.2x + 15.149) ; r2 = 0.99
Arrhenius r2 = 0.925
ln(µ)
Temperature(-1/°K)
a
Ratkowsky function
Experimental data
r2 = 0.925
Square root µ
Temperature °K
b


1e+8
1e+7
1e+6
1e+5
13 °C
20 °C
30 °C
Bacillus cereus cell # (CFU.mL-1)
0 1 2 3 4 5 6 7 8 9 10
Time (h)

1


Test 13°C
Control 13°C
a
Test 20°C
Control 20°C
b
Test 30°C
Control 30°C
c
Aer. hydrophila cell # (CFU.mL-1)
Aer. hydrophila cell # (CFU.mL-1)
Aer. hydrophila cell # (CFU.mL-1)
1.2e+6
1.0e+6
8.0e+5
6.0e+5
4.0e+5
2.0e+5
0.0
0 1 2 3 4 5 6 7 8 9 10
Time (h)

2

3

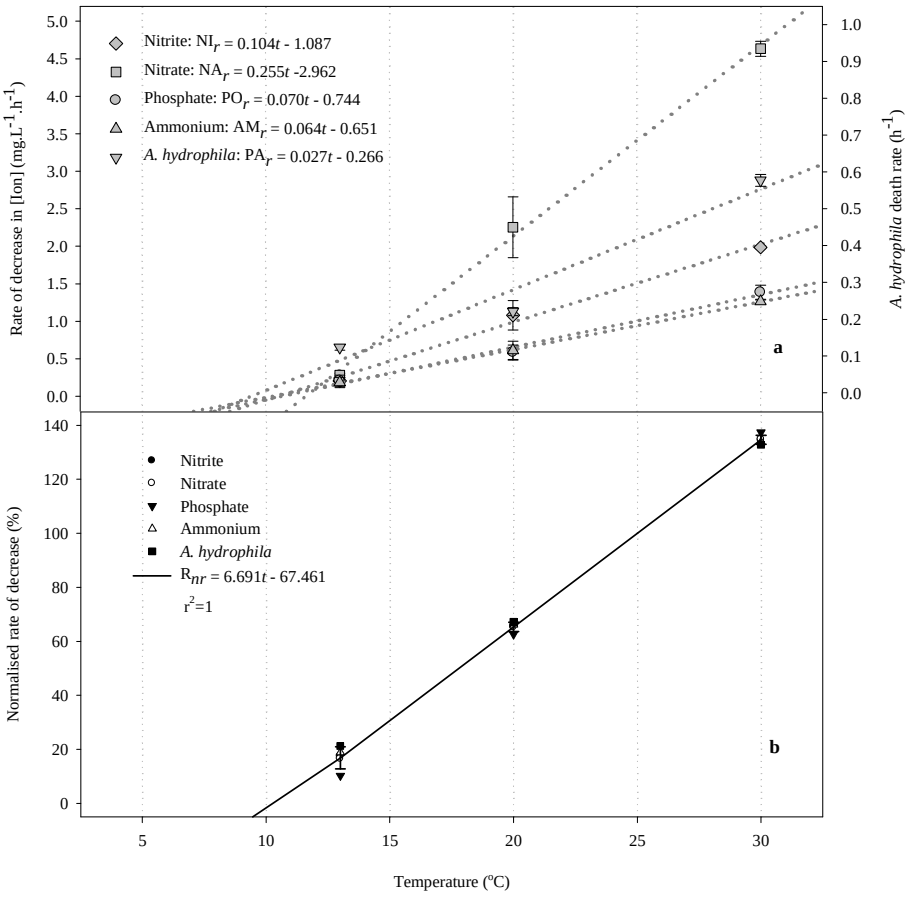
4

5

6

7

8


Nitrite: NI$_r$ = 0.104t - 1.087
Nitrate: NA$_r$ = 0.255t -2.962
Phosphate: PO$_r$ = 0.070t - 0.744
Ammonium: AM$_r$ = 0.064t - 0.651
A. hydrophila: PA$_r$ = 0.027t - 0.266
Rate of decrease in [Ion] (mg.L^{-1}.h^{-1})
A. hydrophila death rate (h^{-1})
a
Nitrite
Nitrate
Phosphate
Ammonium
A. hydrophila
R$_{nr}$ = 6.691t - 67.461
r^2=1
Normalised rate of decrease (%)
b
Temperature (°C)